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Osmotic Tolerance Limits and Effects of Cryoprotectants on Motility of Bovine Spermatozoa[1]

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ABSTRACT

This study was conducted to determine the osmotic properties of bull spermatozoa, including the effects of osmotic stress and cryoprotectant agent (CPA) addition and removal, on sperm motility. Semen from beef bulls was collected by electroejaculation and extended 1:3 in TL-Hepes containing 100 μg/ml pyruvate and 6 mg/ml BSA. In solutions of 150–1200 mOsmolal (mOsm), bull spermatozoa behaved as linear osmometers (r^2 = 0.97) with an osmotically inactive cell volume of 61%. The isosmotic cell volume was 23.5 μm^3. Motility was determined after exposure to anisosmotic solutions ranging from 35 to 2400 mOsm and after return to isosmotic conditions. Retention of at least 90% of isosmotic motility could be maintained only between 270–360 mOsm. Bull spermatozoa were calculated to retain 90% of their isosmotic motility at 92–103% of their isosmotic cell volume. Motility following a one-step addition and removal of 1 M glycerol, dimethyl sulfoxide, and ethylene glycol was reduced by 31%, 90%, and 6%, respectively, compared with CPA addition only. These data indicate that, during bull spermatozoa cryopreservation, osmotically driven cell volume excursions must be limited by exposure to a very narrow range that may be facilitated by the use of ethylene glycol as a CPA.

gamete biology, sperm, sperm motility and transport

INTRODUCTION

Cryopreserved bull spermatozoa have been used since the 1950s for insemination of dairy cattle [1, 2]. Many different extender media with varying concentrations of glycerol, equilibration times, and cooling conditions have been established and found to be effective for bull sperm cryopreservation. However, up to 60–70% of bull sperm are killed by current methodologies [3, 4]. In addition, many bulls produce spermatozoa that do not survive using current cryopreservation protocols [2]. In order to facilitate improvements in cryopreservation of bull spermatozoa, an understanding of the underlying fundamental cryobiological properties of these cells is needed, including their osmotic tolerance limits and response to the addition and removal of permeating cryoprotective agents (CPAs).

Of critical importance in the development of cryopreservation protocols is the method in which CPAs are added before freezing and removed after warming [5, 6]. While CPAs are essential for cell survival during cryopreservation, their addition and subsequent removal creates an anisomotic environment for the cells, resulting in osmotically driven volume excursions and potential osmotic damage. During the addition of a permeating CPA to a cell suspension, the cells are exposed to a hyperosmotic environment. The cells first shrink as water leaves through the plasma membrane and then swell as the CPA enters the cell and water reenters to maintain chemical potential. During the removal of a CPA, cells will initially swell due to an influx of water and then slowly return to isosmotic volume as CPA and water leave the cell. The repeated changes in solution osmolality can result in a significant loss of functional integrity, such as sperm motility, or even cell death without loss of plasma membrane integrity [5]. In addition to causing osmotic damage, CPAs can cause loss of cell viability due to true chemical toxicity. In this regard, a loss of sperm motility can result from prolonged exposure to CPAs, and sperm of different species vary with respect to the type of and duration of exposure to CPAs they can tolerate [7].

Before an optimized procedure for addition and removal of CPAs can be designed, the osmotic tolerance limits of the specific cell type must be determined so that cells will be maintained in a volume range compatible with maintenance of functional integrity [5]. In addition, other cell characteristics must be known, including the isosmotic cell volume, the osmotically inactive cell volume, the hydraulic conductivity or water permeability of the cell membrane (L_P), and the membrane permeability coefficient for the CPA (P_{CPAs}). In the many years of bull spermatozoa cryopreservation, these biophysical and osmotic properties of bull sperm have not been rigorously investigated.

Therefore, a series of experiments was conducted with bull spermatozoa to 1) determine the isosmotic cell volume (V_{iso}), 2) determine cell osmotic response and osmotically inactive fraction (V_b) of cell volume, 3) confirm that bull spermatozoa respond to anisosmotic solutions as linear osmometers, 4) determine osmotic tolerance limits, and 5) determine the effects of one-step addition and removal of CPAs on motility.

MATERIALS AND METHODS

Spermatozoa Processing

Semen used in this study was collected by electroejaculation from fourteen 1- to 2-yr-old Angus and crossbred bulls that were being tested for semen quality at the Purdue University Veterinary Clinic or at the Purdue University Beef Farm. The extender was a Tyrode lactate Hepes buffered medium (TL-Hepes) [8] containing 6 mg/ml BSA and 100 μg/ml of py-

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ruvate (288 ± 5 mOsmolal [mOsm]). The medium was prewarmed to 37°C, semen was extended 1:3, and diluted semen was transferred into an insulated thermos containing water at 37°C for transport to the laboratory within 2 h of collection. The extended semen was centrifuged at 400 × *g* for 5 min and resuspended in a volume calculated to yield a concentration of 1×10^9 spermatozoa/ml. All experiments were conducted at room temperature, 22–24°C.

Media

Hyposmotic TL-Hepes solutions were prepared by diluting isosmotic TL-Hepes with Millipore purified water (3 mOsm) to produce solutions of 35, 75, 150, 225, and 270 mOsm. Hyperosmotic TL-Hepes solutions were prepared by dissolving NaCl in TL-Hepes to make solutions that were 350, 370, 425, 600, 1200, and 2400 mOsm. Anisosmotic PBS solutions were prepared by dilution of PBS or 10× PBS to 150, 290, 600, and 1200 mOsm. Solutions of glycerol, dimethyl sulfoxide, and ethylene glycol were prepared at a concentration of 4 M in TL-Hepes. All materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Osmolalities were determined using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT).

Experiment 1: Cell Osmotic Response and V_b

Osmotic behavior of bull spermatozoa was determined by an electronic particle counter. A Coulter counter ZM model (Coulter Electronics, Hialeah, FL) with a 50-μm standard-resolution aperture tube was used to determine cell volume as described previously [9]. Sperm cell volumes were calibrated for each anisosmotic solution using spherical styrene beads (Duke Scientific Corporation, Palo Alto, CA) with a diameter of 3.98 ± 0.03 μm and a volume of 33.0 μm^3. The Coulter counter was interfaced with a microcomputer using a CSA-1S interface (Great Canadian Computer Co., Edmonton, AB, Canada). The changes in cell volume over time were recorded using a computer software program [9]. Cell volume estimates were based on the modal value of the distribution of the osmotically active population.

Isosmotic cell volume was determined by adding 100 μl of a sperm cell suspension to 15 ml of isosmotic PBS (290 mOsm), and cell volumes were measured in triplicate at room temperature. Osmotically driven cell volume responses and V_b were determined by adding 100 μl of a cell suspension in isosmotic PBS to 15 ml of anisosmotic solutions of PBS (150, 600, and 1200 mOsm). The cell volumes were measured using the particle counter 5–10 min after dilution. To confirm that bull sperm behave as linear osmometers, equilibrium cell volumes were fitted to the reciprocal of the extracellular osmolality of the solution, which is described by the Boyle van't Hoff relationship,

$$V/V_{iso} = (M_{iso}/M)(1 - V_b/V_{iso}) + (V_b/V_{iso}),$$

where V is the cell volume at osmolality M, V_{iso} is the cell volume at isosmolality (M_{iso}), and V_b is the osmotically inactive cell volume (including both cell solids and osmotically inactive water). Spermatozoa from four bulls were used (n = 4).

Experiment 2: Osmotic Effects on Bovine Spermatozoa Motility

The osmotic tolerance of bull spermatozoa using motility as the endpoint was determined using two osmotic stress treatments: 1) exposure of cells to a series of anisosmotic TL-Hepes solutions and 2) measurement after returning the cells to isosmotic conditions [9–11]. A 10-μl aliquot of each sperm suspension (six bulls; n = 6) was transferred into a 1.5-ml Eppendorf centrifuge tube containing 150 μl from each of the 10 anisosmotic TL-Hepes solutions, yielding a final concentration of approximately 60×10^6 spermatozoa/ml. Motility was determined after a 5-min incubation at room temperature using a computer-assisted analysis system (CASA, model HT M2030, Hamilton Thorne, Beverley, MA). A 7-μl aliquot from each anisosmotic treatment was transferred to a Makler stage, preheated to 37°C, and allowed to equilibrate for 30 sec. A minimum of 200 cells was analyzed in 6–9 fields. Motility estimates were validated manually by the video playback option of the Hamilton Thorne instrument.

Spermatozoa were returned to near isosmolality (270–328 mOsm) by transferring a 100-μl aliquot from each anisosmotic sample into a 1.5-ml Eppendorf centrifuge tube containing 200 μl of TL-Hepes solutions at osmolalities calculated to restore cells to an isosmotic environment [9]. Motility was determined after a 5-min incubation at room temperature.

Experiment 3: Effects of One-Step Addition of CPAs and Equilibration Time on Motility

A 10-μl aliquot of the sperm suspension from each of three bulls (n = 3) was transferred into a series of 1.5-ml Eppendorf centrifuge tubes containing no CPA, glycerol, dimethyl sulfoxide, or ethylene glycol to a final volume of 200 μl in TL-Hepes and a final CPA concentration of 0, 1, or 2 M. Samples were incubated at room temperature for 5, 10, and 20 min, and then the motility of each sample was determined as described in experiment 2.

Experiment 4: Effects of One-Step Addition and Removal of CPAs on Motility

In this experiment, semen from five bulls (n = 5) was exposed to the same CPA concentrations used in experiment 3. Motility was determined after a 5-min exposure to CPAs. Then the CPAs were removed from the spermatozoa by centrifuging the samples (400 × *g* for 5 min) and resuspending the sperm pellets in isosmotic TL-Hepes (190 μl) to a final volume of approximately 200 μl. Motility was determined after a 5-min incubation. After each sperm motility determination, the solution osmolality was determined in 10-μl aliquots of each sample.

Statistical Analysis

For experiment 1, V_b was determined as the Y intercept of the regression of the normalized cell volume versus 1/normalized osmolality.

Motility data were analyzed using the mixed model analysis of variance procedure in the Statistical Analysis System, version 6.12 [12], software. In experiment 2, motility data for the two osmotic stress treatments (anisosmotic versus return to isosmotic) were normalized independently to their motility at 290 mOsm. The fixed effects were osmotic stress treatment, eight levels of osmolality, and their interactions in a 2 × 8 factorial treatment arrangement with bull as the random effect. No evidence of variance heterogeneity was found among treatments. In experiments 3 and 4, data were normalized to the motility value recorded after 5 min in the absence of CPAs. The experimental design for experiment 3 was a mixed effects model with a 3 × 2 × 3 factorial treatment arrangement with three types of CPAs, two concentrations of CPAs (1 and 2 M), and three sperm-CPA equilibration times. The experimental design for experiment 4 was a mixed effects model with 3 × 2 × 2 factorial treatment arrangement with three types of CPAs, two concentrations of CPAs, and two CPA manipulations (one-step addition versus one-step removal). Bull was the random effect in experiments 3 and 4.

RESULTS

In experiment 1, the isosmotic cell volume of bull sperm was calculated to be 23.5 μm^3. Analysis of cell volume in anisosmotic conditions indicated that bull sperm were linear osmometers in the range of 150–1200 mOsm ($r^2 = 0.98$) and that 61% of total cell volume was osmotically inactive (total solids plus nonosmotically active water, $V_b = 0.61$). A Boyle van't Hoff plot for the means of three bulls is illustrated in Figure 1 by plotting experimentally measured normalized cell equilibrium volume (V/V_{iso}) versus the reciprocal of normalized osmolality (M_{iso}/M).

In experiment 2, motility was determined following osmotic stress treatments: first, exposure to a range of 35–2400 mOsm TL-Hepes solutions and, second, a return to isosmotic TL-Hepes. Figure 2 shows the percentage of motile spermatozoa, normalized to motility at 290 mOsm, plotted as a function of osmolality (35–600 mOsm) for the two osmotic stress treatments: exposure to anisosmotic conditions and after the return to isosmotic conditions. Change in osmolality was a significant source of variation in motility ($P = 0.0001$). Sperm motility decreased abruptly when cells were incubated in a hypo- or hyperosmotic solution. No cells were motile after exposure to either 35 or 2400 mOsm. The pattern of motility change for the two osmotic stress treatments did not differ; there was no significant main effect ($P = 0.2550$) or interaction between osmotic stress and level of osmolality ($P = 0.3669$). There-

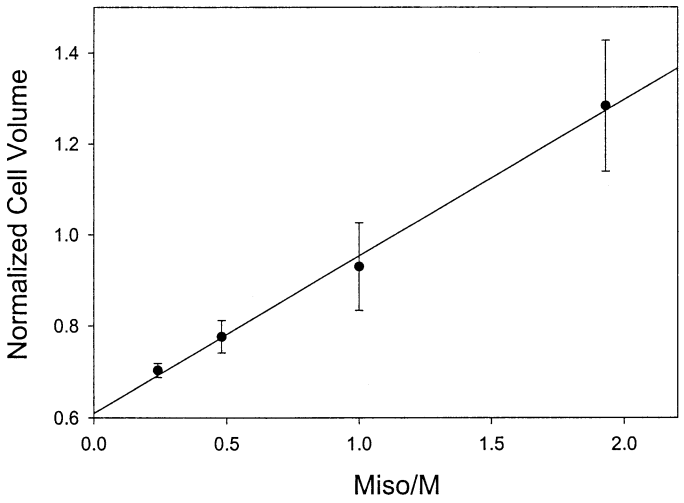

FIG. 1. Boyle van't Hoff plot (normalized cell volume versus reciprocal of osmolality) of spermatozoa from three bulls derived from equilibration volumes exposed to PBS solutions of four different osmolalities: 1200, 600, 290, and 150 mOsm (mean ± SD). The *Y* intercept indicates the osmotically inactive water volume (V_b), which is 61% of the isosmotic volume.

TABLE 1. Percent (mean ± SEM; n = 3) motility of bull spermatozoa after exposure to cryoprotectants (CPA).[a]

Cryoprotectant type and concentration	Equilibration time (min)		
	5	10	20
Control (no CPA)	100 ± 0[b]	93.0 ± 9.1	102.2 ± 4.8
Glycerol (1 M)	75.4 ± 12.7	89.5 ± 12.8	77.9 ± 14.6
Glycerol (2 M)	49.6 ± 13.7	47.7 ± 11	59.4 ± 12.4
DMSO[b] (1 M)	76.3 ± 4.7	75.1 ± 6.8	80.9 ± 7.6
DMSO (2 M)	59.6 ± 7.4	64.9 ± 6.7	63.1 ± 8.8
Ethylene glycol (1 M)	62.1 ± 7.3	82.4 ± 4.8	68.8 ± 6.3
Ethylene glycol (2 M)	44.7 ± 8.6	47.0 ± 15.9	53.6 ± 12.9

[a] Sources of variation: CPA initial concentration ($P = 0.0001$), type of CPA ($P = 0.2549$), and equilibration time ($P = 0.4962$); none of the two- or three-way interactions were statistically significant ($P > 0.05$).
[b] Dimethyl sulfoxide.

fore, the pattern of motility inhibition caused by hypo- and hyperosmotic TL-Hepes was not reversed following the return of the spermatozoa to isosmotic TL-Hepes. Extrapolating from the means of the two treatment curves in Figure 2, the range in osmolality that was consistent with the maintenance of at least 90% of isosmotic motility was 270–360 mOsm. Using electronic particle sizing data from experiment 1 and the motility curve data (Fig. 2), as previously described [10], a volume change in the range of 92–103% was found to be compatible with retention of 90% of the motility measured in an isosmotic environment.

Experiment 3 was conducted to determine motility following exposure to three different CPAs, two CPA concentrations, and three durations of CPA exposure. The results are shown in Table 1. The concentration of CPA had a significant effect on motility ($P = 0.0001$). Actual motility (nonnormalized) was approximately 60% in the absence of CPAs and decreased by about 50% after exposure to 2 M CPA. The three CPAs did not differ ($P = 0.2549$) with respect to their inhibitory effects on motility. Likewise, the effect of duration of exposure to CPAs on motility (5, 10, or 20 min) was not significant ($P = 0.4962$). None of the interactions among the main effects were significant ($P > 0.05$).

Experiment 4 was conducted to extend the results of experiment 3 and determine the effects of one-step addition and removal of CPAs on sperm motility. Motility data are shown in Table 2. All samples including controls showed a decrease in motility with manipulation or centrifugation. Most of the reduction in motility for the control samples was associated with two bulls. The factor ''CPA manipulation'' includes sperm manipulation (pipetting and centrifugation), which cannot be avoided. We expect sperm manipulation to be a component of the negative effect on motility for all treatments. While all of the main effects were significant sources of variation in motility (shown in Table

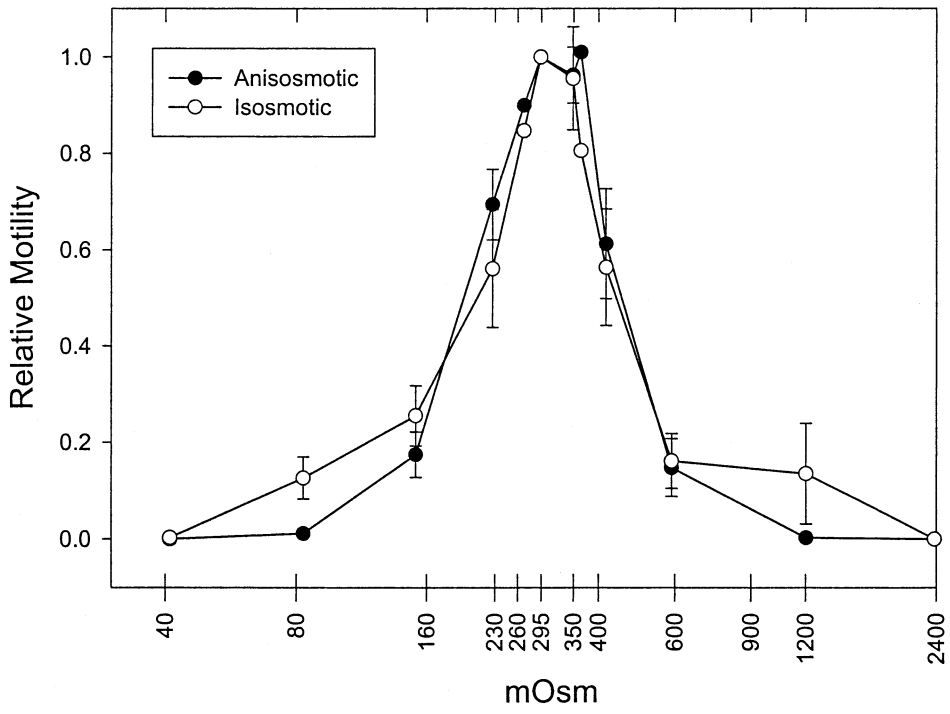

FIG. 2. Percent motility (mean ± SEM; n = 8) of bovine spermatozoa (normalized to isosmotic values for each curve) that were abruptly (in one step) exposed to different osmotic conditions and abruptly returned to near isosmotic conditions as described in *Materials and Methods*. Sources of variation were osmolality ($P = 0.0001$), osmotic stress treatment ($P = 0.2550$), and osmolality by osmotic stress treatment interaction ($P = 0.3669$). Osmolality is in log scale.

TABLE 2. Percentage (mean ± SEM; n = 5) of motile bull spermatozoa as a function of cryoprotectant (CPA) concentration, addition, and removal.[a]

Cryoprotectant type and concentration	CPA present	CPA removed
None (control)	100 ± 0	68.9 ± 7.3
Glycerol (1 M)	63.1 ± 7.1	43.6 ± 3.8
Glycerol (2 M)	53.9 ± 8.0	28.1 ± 8.0
DMSO[b] (1 M)	63.9 ± 4.0	6.4 ± 1.0
DMSO (2 M)	69.0 ± 9.0	2.0 ± 0.8
Ethylene glycol (1 M)	70.4 ± 4.2	66.2 ± 6.6
Ethylene glycol (2 M)	63.8 ± 4.6	50.1 ± 7.4

[a] Sources of variation: CPA type ($P = 0.0001$), CPA manipulation (addition versus removal) ($P = 0.0001$), CPA initial concentration ($P = 0.01160$, and the interaction of CPA type and CPA manipulation ($P = 0.00010$; the other two-way interactions and the three-way interaction were not statistically significant ($P > 0.05$).
[b] Dimethyl sulfoxide.

2), the interaction of CPA type and CPA addition and removal ($P = 0.0001$) was most important to the interpretation of the results. Motility was inversely related to the initial concentration of CPA, as shown in experiment 3. The reduction of CPA concentration to essentially zero by a one-step dilution with TL-Hepes caused a further decrease in motility compared with that after one-step addition of the CPAs. However, the inhibitory effect on motility differed among CPAs, with decreasing inhibitory effect in the following order: DMSO, glycerol, and ethylene glycol. For example, when 1 M CPA was replaced with TL-Hepes, motility was decreased by 90%, 31%, and 6% for DMSO, glycerol, and ethylene glycol, respectively, compared with the presence of CPA. In the case of dilution of 2 M CPA with TL-Hepes, motility was decreased by 97%, 48%, and 22% following removal of DMSO, glycerol, and ethylene glycol, respectively, compared with motility after CPA addition.

Sample osmolality increased to approximately 1300 and 2400 mOsm when spermatozoa were suspended in 1 and 2 M CPA solutions in isosmotic TL-Hepes. The replacement of CPAs with isosmotic TL-Hepes returned the spermatozoa to an environment in which the mean osmolality ranged from 270 to 328 mOsm, with no significant differences with respect to CPA type or initial concentration.

DISCUSSION

An important result of this study was the determination of the relationship between changes in bull sperm cell volume and the osmolality of the surrounding medium. The mean modal volume of isosmotic bull sperm cells obtained from electronic particle sizing, 23.5 μm^3, was similar to volume estimates previously measured for bull spermatozoa based on microscopic measurements, 25 μm^3 [13], and electronic particle sizing values of 25.5 μm^3 [14] and 19–23 μm^3 [15]. Bull sperm are known to behave as linear osmometers [3, 16, 17], and our results extend the range of this behavior to 150–1200 mOsm and show that 61% of the total cell volume is osmotically inactive, i.e., composed of solids and nonosmotically active water. This V_b for bull spermatozoa is greater than that for human (50%) [10], similar to that for the mouse (60.7%) [11], and less than that for the boar (67.4%) [18].

While static changes in bull sperm volume as a function of osmolality were measured using electronic particle sizing, we were unable to detect kinetic volume changes in bull sperm either after exposure of the cells to anisosmotic NaCl solutions or CPA-containing solutions. It appeared that this was the case because the temporal resolution of the Coulter counter measurements was insufficient to monitor the very rapid volume changes exhibited by bull spermatozoa. These measurements are required for the estimation of membrane transport parameters such as L_P, a measure of the rate of water flux across the cell membrane [19], the membrane permeability to CPAs (P_{CPA}), and sigma, which can be derived using the Kedem-Katchalsky membrane transport model [20]. The inability to determine these membrane permeability coefficients for bull sperm was in contrast with our previous studies in human [9] and porcine spermatozoa [16, 21] using electronic particle sizing. In another series of experiments, we tested the hypothesis that the changes in cell volume occurred too rapidly to be measured by the electronic particle sizing technique at room temperature. We attempted to reduce the permeability of cells, and thereby slow the rate of change in cell volume, by adding spermatozoa to hypotonic NaCl solutions and CPA-containing solutions cooled to 15 or 5°C, as described previously [9, 22]. None of these experiments permitted us to measure kinetic changes in cell volume. These experiments are consistent with the hypothesis that this failure to detect kinetic volume changes in bull sperm may have been a result of limited temporal resolution of the Coulter Counter approach. This suggests that the membrane permeability coefficients are relatively high and consistent with previous estimates of bull sperm L_P of 10.8 $\mu m/min/atm$ using a time-to-lysis approach [23].

Another technique, stopped-flow spectroscopy, has been used to measure rapid changes in cell volume in response to osmotic change [24]. Preliminary analyses of bull sperm by stopped-flow spectroscopy permitted measurement of kinetic volume changes in bovine spermatozoa. Thus, it should be possible in future experiments to determine L_P and P_{CPAs} coefficients for bovine spermatozoa.

A fundamentally important result of this study was the determination of the relationship of changes in bull sperm motility to cell volume excursion. These osmotic tolerance data can be utilized in the design of protocols to optimize the addition and removal of CPA at specific temperatures [5]. In this regard, there were two important findings concerning the motility response of bull sperm to osmotic stress. First, bull sperm motility is very sensitive to changes in extracellular osmolality, being intolerant to cell volume excursions induced by exposure to either hypo- or hyperosmotic solutions. In order to maintain motility at 90% of its original isosmotic value, bull sperm must be maintained within 92–103% of their isosmotic volume. Second, the loss in motility in response to hypo- or hypertonic solutions was irreversible when the spermatozoa were returned to an isosmotic environment. Among species investigated in our laboratory, spermatozoa differ in their ability to tolerate the initial anisosmotic exposure and in their ability to recover some level of motility following a return to isosmotic conditions [10, 11, 21]. The differences in the relationship between motility and osmolality for bovine, porcine, murine, and human spermatozoa following exposure to anisosmotic conditions and the return to isosmotic conditions are illustrated in Figure 3. Murine and human spermatozoa are much more tolerant of osmotic stress, especially hyperosmotic conditions (induction of shrinking) than bull and boar sperm and regain a portion of their motility when returned to isosmotic conditions. Murine and human spermatozoa could maintain ≥90% motility for osmotic volume excursions ranging from 90 to 103% [11] and from 75 to 110%

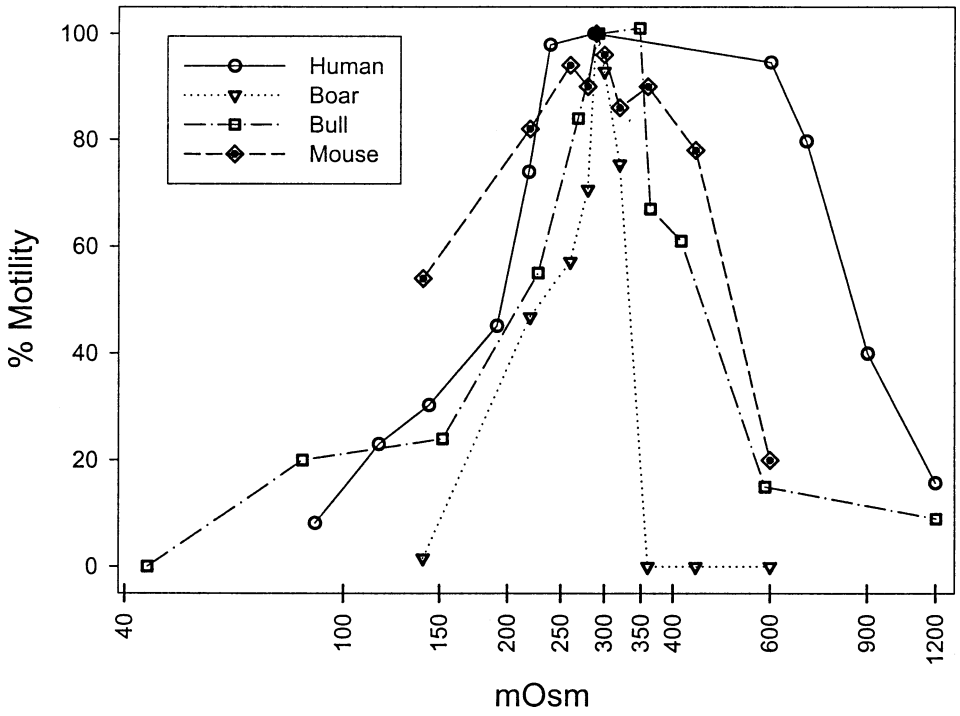

FIG. 3. A comparison of estimated normalized percentage of motilities of spermatozoa of human [10], mouse [11], boar [21], and bovine (current study) after exposure to anisosmotic conditions and return to isosmotic conditions. Osmolality is in log scale.

[10] of their normal isosmotic volume, respectively. Porcine sperm are the least tolerant of osmotic stress and could only maintain 70% of isosmotic motility in a very small volume excursion range of 97–102% of normal isosmotic volume [21]. The response of bull sperm to a return to isosmotic conditions was similar to that of boar sperm in that motility lost during exposure to hypo- and hyperosmotic conditions was not restored following return to isosmotic conditions.

The traditional cryoprotectant used for cryopreservation of bull spermatozoa is glycerol. We found that the one-step addition of glycerol, ethylene glycol, or DMSO resulted in a similar concentration-dependent depression of motility by 40–50% in 2 M CPAs. However, the response to one-step removal differed among the CPAs. Removal of ethylene glycol produced the least harmful effect on motility of the three CPAs, with a further depression in motility of only 6% and 22% relative to one-step addition at the 1 and 2 M concentrations, respectively. Removal of DMSO was quite harmful, with 90% or more of motility lost at 1 and 2 M concentrations. Glycerol produced an intermediate result. When sperm cells in CPA-containing medium are abruptly added to isosmotic solutions free of CPAs, they are exposed to a relatively hyposmotic environment; initially, water enters the cells, causing increased cell volume, and subsequently, CPAs and water exit the cells, resulting in cell volume reduction [5]. The rate of cell volume change for a particular CPA is dependent on the magnitude of the L_P in the presence of CPAs and the P_{CPAs} coefficient [5]. The effect of one-step removal of CPAs on motility in this study followed the order established for sperm motility and P_{CPAs} coefficient magnitude reported in other species; the order of decreasing inhibition was ethylene glycol, glycerol, and DMSO at 22°C in swine [21] and human [22] spermatozoa.

In this study, sperm motility was inhibited by 25% and 50% at 1 and 2 M CPAs, 1300 and 2400 mOsm, respectively. However, this level of motility inhibition was small compared with the almost complete suppression of motility using NaCl to increase osmolality to 1200 and 2400 mOsm in experiment 2. On an equal osmolality basis, permeating CPAs create less cell volume excursion (in magnitude and duration) because the CPAs traverse the plasma membrane [5].

In conclusion, of the millions or billions of spermatozoa normally used to inseminate different species of farm livestock, only a tiny fraction of the sperm reach the site of fertilization. The differences among species in the ability of their spermatozoa to survive cryopreservation may be related to their tolerance of osmotic stress [5]. Therefore, it is of critical importance that the osmotic behavior of spermatozoa be determined and that cryopreservation protocols be adjusted so that it is possible to inseminate with sperm with motility and survivability as high as possible. The relatively less harsh effects of ethylene glycol on motility lead us to suggest that, in future experiments, ethylene glycol should be evaluated as an alternative to glycerol for bull sperm cryopreservation.

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